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24 JUL 1998

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## Protection of Plants

### OBJECT OF THE INVENTION

The present invention describes a new species of binucleated *Rhizoctonia* suited for use in plant protection.

### SUMMARY OF THE INVENTION

The present invention describes a new species of binucleated *Rhizoctonia* able to protect different kind of plants, like tomatoes, onions, grass, pine trees, and others, from infections produced by different pathogenic fungi among them *Fusarium*, *Verticillum*, *Phytophthora* and pathogenic *Rhizoctonia*, as well as yeast and pathogenic bacteria and the appropriated methodology for the molecular identification of this new species, its detection based on probes specially designed and its practical application for the protection of crops.

Thus the present invention uses the ITS data to distinguish isolates and in particular to identify pathogenic and candidate protective isolates belonging to the binucleated group.

The invention also provides specific PCR primers for the ITS molecular analysis of pathogenic and protective strains of *Rhizoctonia*.

### PREFERRED EMBODIMENTS

The protective species can be utilised in a form of powder obtained after fermentation of the micro-organisms with subsequent drying of the filtered broth , applied to the seed before seeding or applied to the soil before seeding or both , and will preclude the growth of the pathogen *Rhizoctonia* protecting the plant from the infection.

In the present invention, the ITS sequence is employed as a marker to distinguish strains and correspondingly classify the pathogenicity of isolates of *Rhizoctonia*. The ITS information enables phylogenetic location of protective isolates, and design of specific primers for the identification of new isolates.

## SUMMARY OF FIGURES

Figure 1 is a schematic representation of the Internal Transcribed Spacer (ITS) region of ribosomal DNA of *Rhizoctonia*.

Figure 2 is a phylogenetic tree for the results obtained in the Examples.

Figure 3 shows photographic evidence of the protective effect of RBN isolates B and F2 on tomato seedlings.

- 3.a.- Shows the plates corresponding to the tomato seedling as negative control and the plate with tomato seedling with the pathogenic strain Me 8-2.
- 3.b.- Shows the negative control with the protective isolates without pathogen.
- 3.c.- Protective effect of F2 on Me 8-2 action, both simultaneous and subsequently.
- 3.d.- Protective effect of B on Me 8-2 action , both simultaneous and subsequently.

## EXAMPLES OF THE INVENTION

In this study we sequenced the ITS - 5.8S regions of 13 binucleate isolates of *Rhizoctonia* isolated from Albacete (South-East of Spain), 9 binucleate isolates from CBS (Central Bureau Schimmelcultures, Baarn, Delft, Netherlands) collection, 3 multinucleate isolates from Albacete and multinucleate isolates belonging to AG 2. The later multinucleate isolates were included to determine the sequence variation in rDNA regions and to assess this variation as a possible tool for identifying groups into *Rhizoctonia* spp. More generally, the isolates from collections were included for comparison purposes and completeness of the phylogenetic tree.



### Isolation of *Rhizoctonia* spp. from the field.

Samples of saffron, alfalfa and pine seedlings were collected at different locations in Albacete, South East of Spain. Isolation of *Rhizoctonia* spp. from these samples was performed by washing saffron roots, alfalfa stems and roots and whole pine seedlings with tap water to remove adhering soils particles and plating plant segments on 2% water agar amended with chloramphenicol (250 mg/ml). The cultures were characterised as *Rhizoctonia* by observing hyphal and colony morphology and vegetative features microscopically, such as hyphae with dolipore septa, basally constricted branching near the distal septum of cells, and absence of clamp connections and conidia. Cultures were maintained and stored at room temperature on potato-dextrose-agar [PDA, Oxoid LTD, U.K.]. The number of nuclei per hyphal cells was determined using a staining method described by Julian *et al.* (Julian *et al.*, 1997). Briefly, 3 mm<sup>2</sup> agar plugs of inoculum were placed in petri dishes containing liquid complete medium [CM: 5 g malt extract (Merck, Darmstadt, Germany), 5 g yeast extract (Difco, Detroit, USA) and 5 g glucose anhydrous (Merck, Darmstadt, Germany) per liter] and incubated for 3 d at 230 C in the dark. The resulting mycelial colony was transferred to a microscope slide, excess medium was removed and a drop of 50 mg/ml Hoechst 33342 (Molecular Probes Europe, Leiden, The Netherlands) was added. After a 15 min incubation in the dark, the staining solution was removed and 50% glycerol was added. A coverslip was placed on sample and the number of nuclei was determined by fluorescence microscopy using an Olympus BHS microscope supplemented with a BH2-RFC reflected light fluorescence attachment (with dichroic mirrors BH2-DMU for UV excitation).

### Other *Rhizoctonia* isolates

Binucleate *Rhizoctonia* spp. isolates have been obtained from the CBS collection and are listed in Table 2.

Table 1. Original isolates described and sequenced for the first time in the invention

Isolates	Nuclear condition	Identification	Isolated from	Geographical origin
F1	Binucleate	Unknown	Saffron	Albacete (Spain)

F2	Binucleate	Unknown	Saffron	Albacete (Spain)
F3	Binucleate	Unknown	Saffron	Albacete (Spain)
F5	Binucleate	Unknown	Saffron	Albacete (Spain)
F6	Binucleate	Unknown	Saffron	Albacete (Spain)
F7	Binucleate	Unknown	Saffron	Albacete (Spain)
S1	Binucleate	Unknown	Saffron	Albacete (Spain)
S2	Binucleate	Unknown	Saffron	Albacete (Spain)
S3	Binucleate	Unknown	Saffron	Albacete (Spain)
S4	Binucleate	Unknown	Saffron	Albacete (Spain)
S5	Binucleate	Unknown	Saffron	Albacete (Spain)
S6	Binucleate	Unknown	Pinus pinaster	Albacete (Spain)
S7	Multinucleate	Unknown	Pinus pinaster	Albacete (Spain)
A1	Multinucleate	Unknown	Alfalfa	Albacete (Spain)
A2	Multinucleate	Unknown	Alfalfa	Albacete (Spain)
B	Binucleate	Unknown	Alfalfa	Albacete (Spain)

Table 2 Reference isolates belonging to the Binucleated Group obtained from different collections

Isolates	Nuclear Condition	Identification	Isolated in	Geographical Origin
C1	Binucleate	Ceratobas. Cornigerum	Festuca sp.	Pennsylvania USA
C2	Binucleate	Cerarobas. Cornigerum	lead of Pittosporum	Pennsylvania USA

C4	Binucleate	Ceratobas. Cornigerum	Juniperus sp.	North Carolina USA
C5	Binucleate	Ceratobas. Cornigerum	Taxus sp.	Rhode Island USA
C6	Binucleate	Ceratobas. Cornigerum	Erigeron canadiensis	Pennsylvania USA
C8	Binucleate	Ceratobas. Cornigerum	Pittosporum sp.	Florida USA
C11	Binucleate	Ceratobas. Cerealis	Secale cereale	Wageningen, NL
C12	Binucleate	Ceratobas. Cerealis	Secale cereale	Germany
C13	Binucleate	Ceratobas. Cerealis	Triticum aestivum	Germany
17-329	Binucleate AG-A	Ceratobas. Corgigerum	Strawberry	Saga, Japan
17-326	Binucleate AG-A (CAG-2)	Ceratobas. Cornigerum		USDA
16-321	Binucleate AG-BO	Ceratobas. Cornigerum	Patata	Tokushima, Japan
17-328	Binucleate AG-B6			USDA
16-320	Binucleate AG-C	Ceratobas. Cornigerum	Gimnadenia conopsea	Hokkaido, Japan
16-319	Binucleate AG-D	Ceratobas. Gramineum	Wheat	Hokkaido, Japan
4-7 b	Binucleate AG-E	Ceratobasidium sp.	Flax	Hokkaido, Japan
17-331	Binucleate AG-G	Ceratobasidium sp.	Peanut	Chiba, Japan
16-315	Binucleate AG-I	Unknown Artemisia	Tokaydo, Japan	
16-314	Binucleate AG-J	Ceratobasidium sp.	Soil	Kanto, Japan
16-317	Binucleate AG-K	Ceratobasisium sp.	Onion	Hokkaido, Japan

9-363	Binucleate AG-L	Ceratobasidium sp.	Soil	Fukuoka, Japan
19-368	Binucleate AG-N	Unknown	Soil	Tochigi, Japan
19-367	Binucleate AG-P	Ceratobas. Cornigerum	Tea	Shizuoka, Japan
19-365	Binucleate AG-R	Ceratobasidium sp.	Cucumis	Georgia, USA
19-366	Binucleate AG-S(CAG-7)	Ceratobas. sp.	Pinosporum	Florida, USA

Table 3 Isolates of multinucleated *Rhizoctonia solani* AG-2.

Isolates	Nuclear condition	Identification	Isolated in	Geographical origin
J1	Multinucleate L-9-1S	Thanatephorus cucumeris	Zoysia grass	Gifu, Japan
J2	Multinucleate G4	Thanatephorus cucumeris	Zoysia grass	Gifu, Japan
J3	Multinucleate AJ-1-10-1	Thanatephorus cucumeris	Zoysia grass	
J4	Multinucleate RGR38	Thanatephorus cucumeris	Zoysia grass	Shizuoka, Japan
J5	Multinucleate RGR 39	Thanatephorus cucumeris	Zoysia grass	Shizuoka, Japan
J6	Multinucleate SLP3-3	Thanatephorus cucumeris	St. Augustine grass	Okinawa, Japan
J7	Multinucleate RGR-59	Thanatephorus cucumeris	Bent grass	Shizuoka, Japan
J8	Multinucleate C-116	Thanatephorus cucumeris	Mat rush	Kumamoto, Japan
J9	Multinucleate C-306	Thanatephorus cucumeris	Mat rush	
J10	Multinucleate 9207	Thanatephorus cucumeris	Beefsteak plant	Hyogo, Japan
J11	Multinucleate No. 2	Thanatephorus cucumeris	Maize	Miyazaki, Japan
J12	Multinucleate Gu-1	Thanatephorus cucumeris	Guzmania	Gifu, Japan

J13	Multinucleate K-1	Thanatephorus cucumeris	Sugar beet	Hokkaido. Japan
J14	Multinucleate S-2	Thanatephorus cucumeris	Sugar beet	Hokkaido. Japan
J15	Multinucleate R56	Thanatephorus cucumeris	Sugar beet	Hokkaido. Japan
J16	Multinucleate R64	Thanatephorus cucumeris	Sugar beet	Hokkaido. Japan
J17	Multinucleate 92155-1-2	Thanatephorus cucumeris	Burdock	Hokkaido. Japan
J18	Multinucleate 92155-1-3	Thanatephorus cucumeris	Burdock	Hokkaido. Japan

### Pathogenicity assays

Pathogenicity tests were performed with the strains isolated from the field in Albacete on the following plant species: radish (*Raphanus sativus*), cauliflower (*Brassica oleracea* var. *botrytis*), onion (*Allium cepa*), turf grasses: zoysia grass (*Zoysia tenuifolia*), St. Augustine grass (*Stenotaphrum secundatum*) and Bermuda grass (*Cynodon dactylon*), tomato (*Lycopersicum esculentum*), carrot (*Daucus carota*) and cucumber (*Cucumis sativum*). Seeds were surface-disinfected in 1% sodium hypochlorite for 10 min, rinsed with sterile distilled water and aseptically blotted between two layers of wet sterile paper into petri dishes. They were allowed to germinate for 4 days at 260 C in the dark. Isolates were grown on water agar for 2 days at 260 C. Five germinated seeds were then placed around the fungal colony. The plates were incubated at room temperature in the lab bench, and pathogenicity was assayed for the first time 7-10 days after the tests were set, and subsequent observations were made afterwards every two days. The *Rhizoctonia solani* AG 4 strain Me8-2 (Boysen *et al.*, 1996) was used as positive control, since this strain is known to be very pathogenic to all plant species tested. A negative control of germinated seeds without fungus was included in each of the tests.

### Protection assays

The strains that did not show pathogenicity towards one or more plant species were tested for protection from the pathogenic strain Me 8.2. This was done by inoculating both the pathogenic strain and the supposedly protective strain. This was done in two ways, simultaneously (both strains inoculated at the same time) and successively (first the supposedly protective strain was allowed to colonise the plants surface for three days and then the pathogenic strain was inoculated). A negative control (no fungus), a positive control (the pathogenic strain Me 8.2), and the supposedly protective strain alone were included for each of the plant species in the tests. Protection was assayed for the first time 7 days after the tests were set, and subsequent observations were made every two days.

#### Isolation of genomic DNA, asymmetric PCR and DNA sequencing procedures

For DNA extraction, *Rhizoctonia* plugs were inoculated in 50 ml complete medium [CM: 5 g malt extract (Merck, Darmstadt, Germany), 5 g yeast extract (Difco, Detroit, USA) and 5 g glucose anhydrous (Merck, Darmstadt, Germany) per litre] in a flask, the mycelium was grown for 4 days at 23° C and 150 rpm agitation, blotted dry and freeze dried. After grinding, mycelium was suspended in 500 ml DNA-extraction buffer [20 mM Tris/HCl pH 8.0, 50 mM EDTA and 0.4 % (w/v) SDS], incubated at room temperature for 5 min and extracted twice with an equal volume of phenol: chloroform: isoamylalcohol (25:24:1). Subsequently, the aqueous phase was incubated for 30 min at 37° C in the presence of 50 mg/ml RNase A and then extracted with chloroform: isoamylalcohol (24:1). DNA was precipitated by adding 0.6 volumes isopropanol, washed with 70% ethanol, dried and resuspended in 100 ml ultrapure sterile water.

Eucariotical ribosomal genes are arranged in a tandem repeat with the 5.8S gene flanked by internal transcribed spacers (ITS). Fig 1 is a schematic representation of the Internal Transcribed Spacer (ITS) region of the ribosomal DNA. The shadowed boxes represent the ribosomal genes. The arrows denote the position of the PCR and sequencing primers.

The regions of rDNA repeat from the 3' end of the 18S gene to the 5' end of the 28S gene were amplified using the primers ITS 5, 4, 4B and 1F (White et al, Gardes et al). Asymmetric PCR to synthesise ssDNA of the ITS region was done with a 50:1 molar ratio between two primers (Gyllenstein). Fragments of approximately 700 bp were obtained when using *Rhizoctonia*

genomic DNA as a template for PCR amplification of the ITS region. Different combinations of primers were tested for the asymmetric amplification. Asymmetric PCR products using ITS4/ITS5 in the ratio 50:1 were found to give better results when sequencing with ITS1 and ITS3, whereas the combination of ITS1F/ITS4B (also in the 50:1 ratio) was found to give better results when sequencing with ITS2 and ITS4. Manual sequencing was performed using the Sanger's dideoxy method (Sanger *et al.*, 1977).

### Phylogenetic analysis

The sequence data were checked between complementary strands using GCG Fragment Assembly System (Program Manual for the Wisconsin Package, version 8, Wisconsin). The resulting sequences were aligned using the software package CLUSTALW (Thompson *et al.*, 1994) (IntelliGenetics, Inc., Mountain View, CA). All the data were processed for phylogenetic analysis of the nucleotide and gap differences. The distances in the ITS-5.8S rDNA region were determined by Kimura two parameter model (Kimura, 1980), omitting all sites with gaps. Phylogenetic trees were constructed using the maximum-parsimony analysis with the heuristic search of the Phylogeny Using Parsimony Analysis (PAUP) program 3.1.1. (Swofford, 1991) which gaps treated as missing data. The branch and bound algorithm provides an efficient approach to find all the minimal lengths. The trees were rooted with the rDNA sequence of *Trichoderma harzianum*. A bootstrap analysis using 1000 resample of the sequence data was carried out, and using the heuristic search option of PAUP. Trees were also obtained by using the Neighbour-joining method (Saitou and M., 1987) as it is in the program "Neighbour" from the PHYLIP package version 3.5 (Felsenstein, 1993). The topological accuracy was again estimated by the bootstrap method with 1000 replicates. The percentage of bootstrap replicates which yields each grouping as a measure of statistical confidence. A grouping found on 95% of bootstrap replicates was considered statistically significant.

## RESULTS

### Pathogenicity assays

Crucifers (radish and cauliflower) are susceptible to all *Rhizoctonia* isolates tested, regardless of whether they were multinucleated or binucleated. The same was true for onion. Grass, tomato and carrot were susceptible to all multinucleated *Rhizoctoniae* (Me 8.2, aA1, aA2), but were not susceptible to any of the binucleate strains, therefore protection tests were done with these plant species. The strains randomly chosen to test protection from the pathogenic strain Me 8.2 were the binucleate strains aB and F2. Cucumber was susceptible to all strains tested, except for S7, therefore this strain was subsequently tested for protection from the pathogenic strain Me 8.2. The strain Me 8.2 showed significantly stronger pathogenicity than any other strains tested, and this was seen in shorter times needed to attack the plant, and complete killing of the seedling, whereas with other pathogenic strains the seedlings were damaged, but not as much affected as with isolate Me 8.2. In the pathogenicity tests was also observed that in some cases the seeds had not been completely surface sterilised, and in the negative control (no fungus) was observed growth of some fungi other than *Rhizoctonia*, yeast or bacteria, but when *Rhizoctonia* had colonised the seedlings, there was no growth of these contaminants.

#### Protection essays

For grass, tomato and carrot, it was observed that the negative control (no fungus) presented contamination from other fungus, yeast and/or bacteria, whereas the control with protective strains B and F2 were clean, that is, *Rhizoctonia* did not allow any of the contaminants carried in the seed to grow. As expected, plants inoculated with protective strains B and F2 looked completely healthy, whereas plants inoculated with Me 8.2 were heavily injured. In the simultaneous test (that is, protective and pathogenic strains inoculated at the same time) there was no protection of the binucleate strains from the pathogenic Me 8.2. But in the successive test, in which the binucleate strains B and F2 were allowed to colonise the plant surface prior to adding the pathogenic strain, a very clear protective effect was observed, and the plants looked as healthy as the negative control and the control with the binucleate strains alone (Fig. 3). Furthermore, it was also observed that the seedlings covered by the protective strains looked slightly bigger than the negative control, thus pointing to the possibility that there is an increased growth response induced by the non-pathogenic *Rhizoctonia* isolates (Fig. 3). This has already been described by (Sneh *et al.*, 1986). In cucumber, although some protection was seen, we could not observe a clear



protective effect of strain S7, therefore we could not conclude that strain S7 acts as a protective strain in cucumber.

We foresee the same protective behaviour for the entire group of the new species including F2. B protects much better than A1 and A2 which are pathogenic.

Sequencing PCR products from the PCR amplified ITS regions

PCR experiments carried out on the ITS-5.8S rDNA region of 20 isolates of binucleate *Rhizoctonia* spp (including isolates of *Ceratobasidium* from CBS collection and RBN testers) A single band was detected in each amplification reaction when analysed by agarose gel electrophoresis. The amplified DNA was sequenced directly in both strands.

Primers obtained from the sequenced isolates of binucleated *Rhizoctonia* for their identification

Albacete

Ab -1

CCCTATTAAGGGGCA

Binucleated

Bin

CCGTAAAAAAGTCTT

Cerealis

Cer11

CGCGAGAGAGAGGCTGGC

Albacete

Ab+

CCACACAAACCCATTGTATT

Cornigerum

Dorg

AATTGTTTCCTTTGGGCAC

Albacete

Ab-2

GGAACCTTTATTGGAC

Cerealis

Cer12

AATGTAATCGATGTAAACGC

Albacete

Ab-3

CTCAATTTATTTTAAAACGA

The molecular definition of the members of the new species group may be based on several specific sequences of the ITS segment. The sequence CCACACAAACCCATTGTATT of the primer AB+ is common and specific to the group of F2, with the exception of S6 that changes one nucleotide (ATCG instead of ATTG), see pages 3-4 of the manual alignment.

This primer may define the group whereas we cannot define the isolate B. This isolate has a unique property of having cells with two and with four or more nuclei. This characteristic, never described before may be important for the understanding of the relationship between binucleated and multinucleated *Rhizoctonia*.

#### Phylogenetic analysis

The CLUSTALW sequence alignments are shown in Exhibit A.

Reference is also made to the phylogenetic tree of Figure 2. The 5.8S rDNA gene sequence (155 bp) was conserved among the *Rhizoctonia* isolates examined. Among the isolates analysed, the sequence of ITS 1 was more divergent than that of the ITS 2 region. There was 100-81%

sequence homology among the sequences of total ITS regions of all the isolates used in this study. Sequence homology among binucleate isolates varied from 99-85%, and 85-75% between binucleate and multinucleated isolates (AG 2). In phylogenetic analysis a consensus sequence of the ITS regions of 18 AG 2 isolates was used. Phylogenetic analysis separates binucleate and multinucleated in two different clusters. In the multinucleated cluster it is possible to differentiate AG 2 and AG 4, and also subgroups within each AG that correlate with pathogenicity and geographical origin. Binucleate isolates clustered together in an independent branch. It is interesting that Spanish isolates from Albacete cluster differentiated from binucleate isolates from the CBS collection. Multinucleated isolates from alfalfa (A1 and A2) cluster in AG 4 group, with similar features of pathogenicity. In the tree, horizontal lengths in the branches indicate the relative genetic distances between the isolates.

F1, F6, F5, F7, S5, S6, F2, F3 and S1 thus comprise the new species so far. S3 clusters with reference strains and should be similar to AGN, AGP, AGBO, and AGK. S2 and SS are similar to AGL, AGI, AGC and AGD.

## REFERENCES

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EXHIBIT A

## C JSTALW ALIGNMENT

[illegible]





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AGC GATCTCAAAATCAGGT-AGG- ---
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AGD GATCTCAAAATCAGGT-AGG- ---
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THARZIANU -----

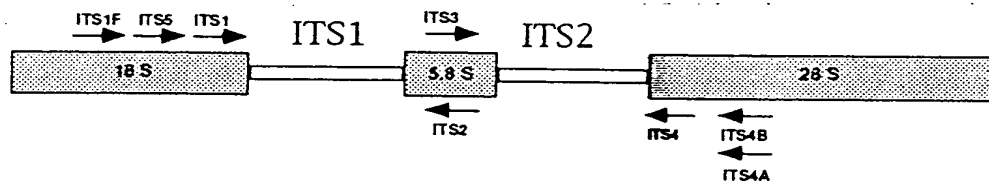
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FIGURE 1

Scheme representing the Internal Transcriber spaces (ITS) region of the ribosomal DNA. The shadowed boxes represent the ribosomal genes. The arrows denote the position of the PCR and sequencing primers.





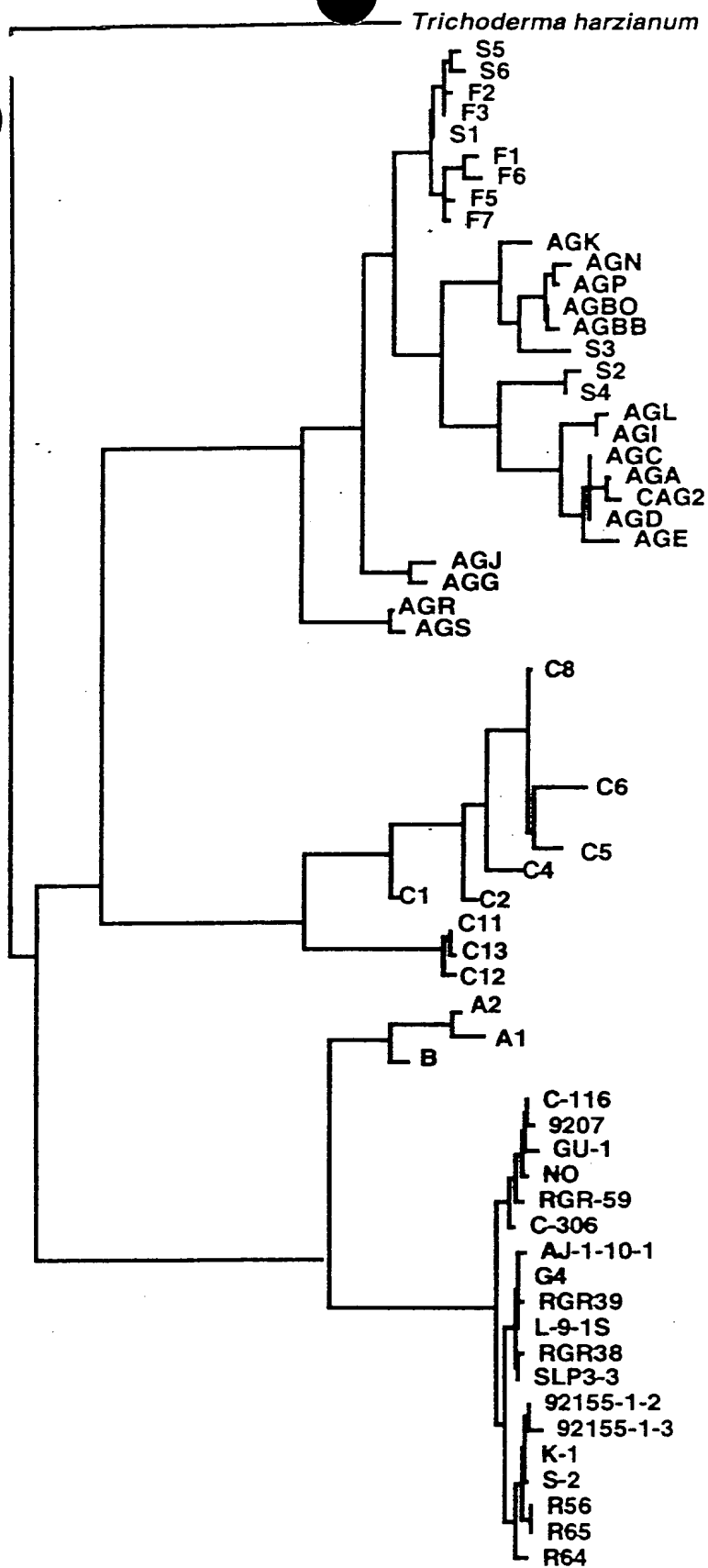


Figure 2



Figure 3

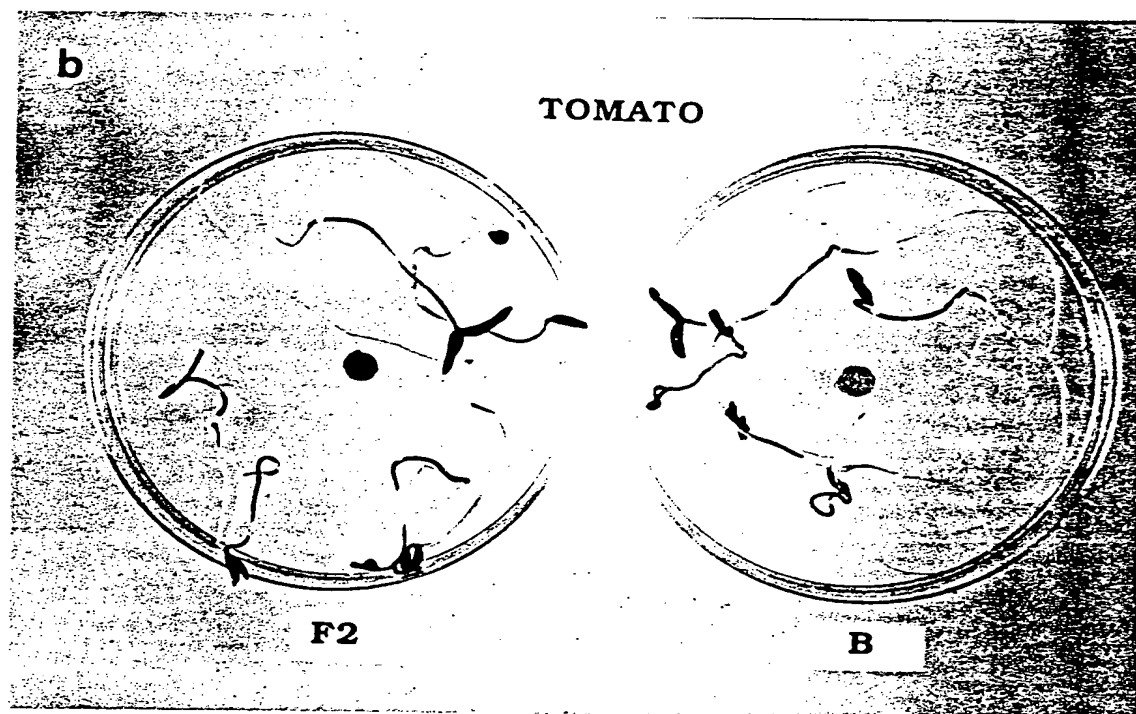
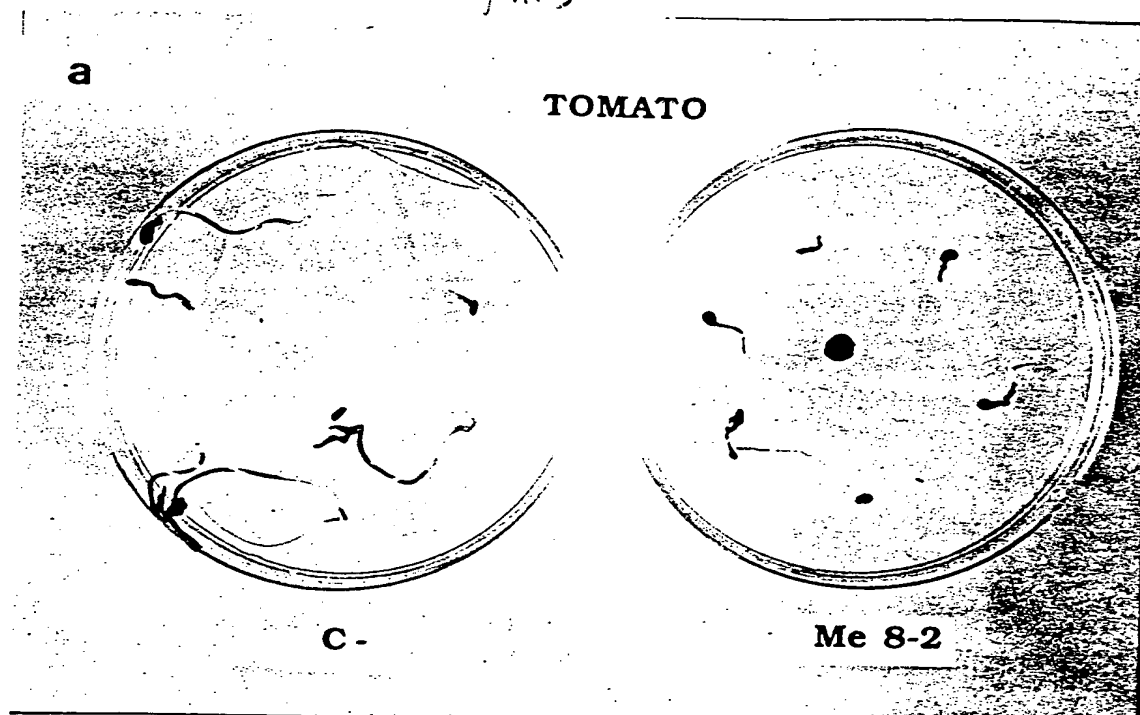
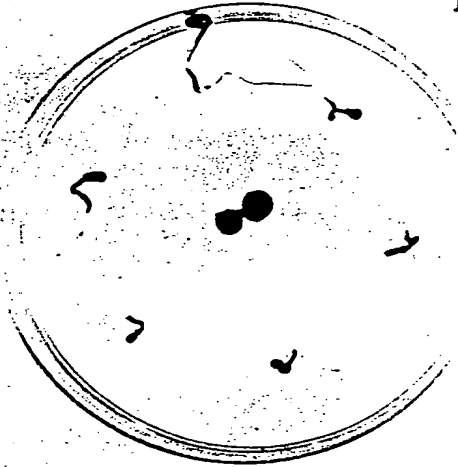




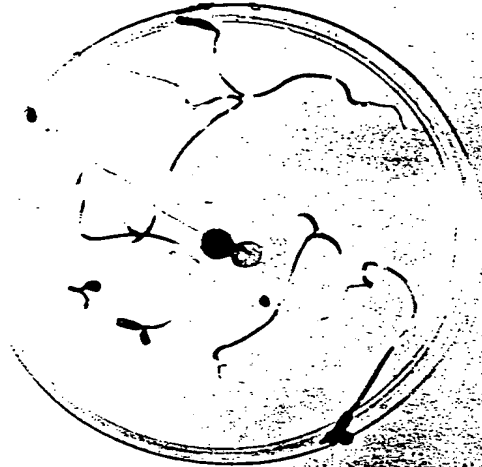
Fig 3

c

TOMATO



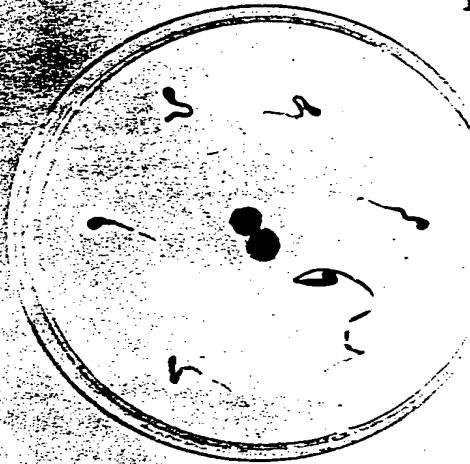
Me 8-2 F2  
simultaneous



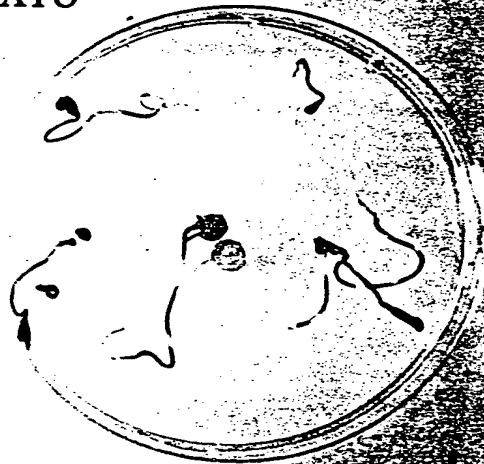
Me 8-2 F2  
consecutive

d

TOMATO



Me 8-2 B  
simultaneous



Me 8-2 B  
consecutive

PCT/GB99/02406

marks + clerk

13/4/99

CP